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14. ABSTRACT We evaluated the threshold for concussive effects of low frequency underwater sound (LFS) exposure. In study 1, anesthetized (sodium pentobarbital 25mg/kg) male Sprague Dawley rats (n=96) were cannulated, ventilated (1.5% isoflurane), submerged, and exposed to 5 min. of LFS using a G40 calibrator. No alteration in cardiovascular function (arterial blood pH, pO2, PCO2, HR, or MABP) was observed during submersion. In study 2, animals were evaluated for two weeks following LFS exposure (150 Hz 180dB, n=12; 250 Hz 194 dB, n=12), submersion only (n=11), or no submersion (n=11) using a battery of neurological motor tests. Cognitive function was assessed at one week using the Morris water maze (MWM). All animals were sacrificed at 15 days following for histological analysis. No effects of LFS on cardiovascular or neurological motor function were observed. Although no adverse cognitive effects of LFS at 150 Hz (180 dB) were observed, animals exposed to 250 Hz (194 dB) LFS exhibited a mild but significant learning deficit. No histological damage associated with LFS could be detected. These data suggest that LFS exposure may cause a mild transient learning impairment when pressure levels exceed 190dB.

15. SUBJECT TERMS

low frequency sound, acoustic trauma, underwater, physiology, behavior, histology, rat

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FINAL REPORT

GRANT #: 00014-97-1-0954

PRINCIPAL INVESTIGATOR: Tracy McIntosh, Ph.D.

INSTITUTION: University of Pennsylvania

GRANT TITLE: CONCUSSIVE EFFECTS OF LOW FREQUENCY SOUND EXPOSURE

AWARD PERIOD: July 1 1997 - 31 March 1999

OBJECTIVE: To evaluate the threshold for concussive and histopathological damage to the CNS following low frequency sound exposure.

APPROACH: Adult male Harlan Sprague Dawley rats (250 -300g, n=96) were placed onto a submersion stretcher and ventilated (2cc x 60-65 breaths/min, Harvard Rodent Ventilator Model 683) with compressed air and 1.0% isoflurane. Animals were randomly assigned to one of following four groups: Sound exposure at 150 Hz and 180 dB (LFS 150), sound exposure at 250 Hz and 194 dB (LFS 250), submersion (underwater) control (U), and a sham submersion (S) group that remained on the table. Duration of the sound exposure was 5 minutes in all experiments. A U.S. Navy, Underwater Sound Reference Detachment's G40 calibrator generated the low frequency underwater sound emissions used in all studies. Each animal except for those in the sham submersion group, was submerged in a head down orientation with the brain positioned ~15 cm from the G40 speaker at a depth of ~25cm from the surface. The sound exposure period was five minutes. To study the physiological effects of LFS, the left femoral artery of the animal (n=36) was cannulated for cardiovascular (HR, MABP) and blood gas (pH, pO2, pCO2) measurements and grouped randomly. Anesthetized, endotracheally-ventilated subjects (n=60) were randomly assigned to one for the four groups described above and were submerged/sham-submerged for 10 minutes. The five-minute acoustic exposure for the two experimental groups began 3 minutes after submersion. Sham-submerged subjects remained ventilated on the table for an equivalent length of time.

Neuroscore

Neurological motor function was evaluated 48 hours, 1 week, and 2 weeks following LFS exposure by an experienced behavioral investigator, blinded to experimental condition. This composite battery of tests was previously described (McIntosh et al., 1989). Briefly, animals were given a score from 0 (severely impaired) to 4 (normal) for 6 individual tests: (1) left and (2) right forelimb flexion during tail suspension, (3) left and (4) right hindlimb flexion when the forelimbs remain on a hard surface and the hindlimbs are lifted up and back by the tail, and (5) left and (6) right lateral pulsion resistance.

Beam Balance

Coordinated motor function was evaluated at 48 hours, 1 week, and 2 weeks following LFS exposure using a beam balance test, described previously (Dixon et al., 1987) & (Hamm et al., 1992). Animals were placed on a narrow wooden beam (1.5 cm wide) for 60 seconds. The animal's ability to maintain balance was scored between 1 (no attempt to balance) and 6 (steady posture). The reported score was the average of three trials.

Rotating Pole

All animals were also tested on a rotating pole task, previously described by Ohlsson and Johansson (1995) to evaluate complex motor function and balance, at 48 hours, 1 week, and 2 weeks following LFS exposure. Briefly, this test consists of a pole 70 cm above the ground rotated clockwise with a variable speed electric motor at zero, three, or ten revolutions per minute. The animal was scored from 0-6 for each of three trials at each speed by a method previously described (Mattiasson et al., 1999). The range is from 0, for a rat that falls off the pole, to 6, for a rat that crosses the pole with no foot-slips. The reported score is the average of three trials.

Morris Water Maze

Cognitive function following LFS exposure was evaluated using a Morris water maze (MWM) paradigm (Morris, 1984) that was modified for TBI by Smith et al. (1991). In this paradigm, animals were introduced into a 1m diameter tank filled with 18°C water in successive directions (north, west, south, and east) over a series of 10 trials on each of Day 8 and 9 following LFS exposure or sham procedures. Animals learn the location of a hidden submerged platform using visual cues external to the maze. The time (latency) to find the escape platform was recorded for each trial.

To evaluate the animal's ability to remember the learned visiospatial task, on Day 10 following sound exposure, the platform was removed from the MWM and animals were allowed to swim for 60 seconds to evaluate their memory of the platform location (probe trial). Their swimming patterns were recorded by video and analyzed to assign a score based on the time spent in specific regions of the maze, giving more weight to those regions closest to the prior platform location. An additional group of animals (n=10) was exposed to 150 Hz LFS at 145 dB and tested for learning impairments at one week using the MWM.

Histological Analysis of Cell Death and Dysfunction

Two weeks after LFS exposure, subjects (n=14) were randomly selected for histological analysis (LFS 150 n=5, LFS 250 n=5, control S + U n=4) from the population of animals evaluated behaviorally. Neurons in Nissl stained 6μm thick coronal sections were quantified in areas of the dorsal and ventral hippocampus (dentate hilus, CA1, and CA3), the brainstem, and the cerebellum (Purkinje cells). Additionally, eosinophilic and/or pyknotic cells were counted in consecutive Hematoxylin & Eosin stained sections.

ACCOMPLISHMENTS: Control (S) animals exhibited stable mean pH, pCO2, and pO2 throughout the time course of the experiment suggesting that there was no harmful effect of the intubation and ventilation procedure. No significant differences were observed in pO2 measurements over time between sham-submerged, submerged, and sound exposed groups. However, in all submerged groups, pCO2 increased over time ($p < 0.001$). This effect was significantly greater in 15 minute submerged animals than corresponding sham-submerged animals ($p<0.05$). Using a ventilation rate of 65 breaths/min, blood pH in submerged (U) animals remained within normal parameters and did not differ from the sham-submerged group. LFS exposure had no effect on mean pH, pCO2, and PO2.

The mean arterial blood pressure (MABP) was found to decrease gradually over the time course of the experiment, however, no difference between groups was observed. This effect is likely due to an increase in vertical distance between the MABP transducer and the

animal after submersion. The heart rate of submerged animals was also found not to change over time and no significant differences between groups were observed.

Neuroscore for experimental and control groups was normal for each of the three time points tested. For submerged control (U) animals, the median score was consistently measured at 22 points out of a possible 24 points at 48 hours, one week, and two weeks following the experimental procedure. Control animals that were ventilated but not submerged (S) scored the maximum 24 points at all three time points. LFS 150 Hz animals consistently scored 22 points at each time point whereas LFS 250 Hz animals scored the optimum score at 48 hours and one week. The median score of LFS 250 Hz animals at two weeks following the experimental procedure was consistently 23 points. No differences were observed between groups (one-way ANOVA on ranks).

Similarly, the evaluation of coordinated motor function with the beam balance test revealed normal function in each of the four groups. The median score from each group across the three time points ranked greater than or equal to five on a maximum scale of six. A Kruskal-Wallis test found no statistically significant differences among groups at 48hours after the experimental procedure. At one week following the experimental procedure LFS 150Hz exposed animals had a median beam balance score significantly less than submerged controls ($p= 0.016$), however, the lower score represents a rank considered normal neurologic motor function. No differences were observed between the four groups at two weeks following the experimental procedure.

No significant neuromotor differences between the experimental and control groups were found using the Rotating Pole test at 3 RPM during the two-week observation period. A Mann Whitney one-way ANOVA was implemented to test for significance between groups at each time period ($p=0.429$, $p=0.553$, and $p=0.222$ respectively). Also, no significant differences were observed between groups using the rotating pole test at 0 RPM and 10 RPM, indicating that the subject's ability to traverse the rotating pole was unaffected by LFS exposure.

All animals demonstrated an ability to learn the visiospatial task over the four blocks of five trials of the MWM learning paradigm as evidenced by the decreasing mean latencies over the four blocks of trials. However, by the fourth set of five trials, animals exposed to LFS 250 Hz at 194 dB exhibited a significantly longer mean latency than submerged controls (U), suggesting a mild, but significant learning deficit (one-way ANOVA with post-hoc tests, $p<0.05$). The LFS 150Hz exposed animals exhibited a learning pattern similar to control groups. No significant differences were observed between the LFS 150 group and controls. Performance in the memory test (probe trial) was similar across all groups (one-way ANOVA, $p=0.687$) indicating a normal memory score in the MWM among experimental groups at two weeks following LFS exposure.

Cell counts of Nissl stained sections within the dentate hilus, CA1, and CA3 regions of the hippocampus revealed no significant cell loss in LFS exposed animals when compared to control groups. Quantitation of eosinophillic or pyknotic neurons within these regions on Hematoxylin & Eosin consecutively stained sections also revealed no statistically significant differences ($p < 0.05$) between LFS exposed groups and control animals.

CONCLUSIONS: In the present study we were able to reproducibly expose anesthetized animals to underwater LFS without significant alteration of physiological and cardiovascular parameters. Neither submersion nor

5 minutes of exposure to either LFS 150 Hz or LFS 250 Hz resulted in decreased function on simple (reflex) motor tasks. Animals exposed to either 150 Hz or 250 Hz sound exposure performed equivalently to control animals on the rotating pole and beam balance tests that incorporate more complex motor functions such as balance and coordinated movement. Although, animals exposed to the highest dB level (250 Hz) showed a mild learning deficit in the Morris Water Maze, no differences were detected in the memory scores between the sound exposed groups and controls. Memory function was not adversely affected by sound exposure.

Although a semiquantitative analysis of cell counts suggested 150 Hz sound exposure caused cellular degeneration in the areas of the brain mentioned above, no statistical differences were found between sound exposed animals and controls which may be due to a small number of animals in the LFS exposed groups exhibiting a disproportionately large number of damaged neurons.

Future studies should focus on cognitive dysfunction in animals exposed to LFS 250 Hz to determine a damage threshold (sound pressure level) and evaluate cognitive and neurological motor function in animals exposed to sound at other frequencies than those employed in the current study.

SIGNIFICANCE: Our studies have provided information for the ONR to establish safety guidelines for underwater divers who may be subjected low frequency sound exposure.

AWARD INFORMATION: 1. Dorothy Russell Memorial Medal for Brain Injury Research, British Society of Neuropathologists, London, England, January 1998. 2. The Isabella Rubin award in Brain Injury, Brain Injury Association, Washington, D.C., July, 1998.

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